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# THE QUANTITATIVE DETERMINATION OF CANE SUGAR BY THE USE OF INVERTASE.

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## INTRODUCTION.

The general method now in use for estimating cane sugar is that of Clerget, in which the sugar is hydrolyzed by acid and the resulting invert sugar estimated either by its reduction of Fehling's solution or by the change in optical rotation which results from the hydrolysis. It has long been known that the presence of other hydrolyzable substances with the cane sugar interferes in the determination because the hydrolysis by acid is then not confined to the cane sugar. This method would be greatly improved if some substitute for the acid were known which would hydrolyze the cane sugar without attacking other acid-hydrolyzable substances; it is the purpose of this article to show that the enzym invertase in most cases fulfils this requirement, and that its use to supplement or even replace acids in the quantitative estimation of cane sugar is of great value in analytical work. The use of this enzym in studying the sugars which occur so widely distributed among plants is particularly recommended.

## THE PREPARATION OF INVERTASE.

To prepare a stock solution of invertase, break up 5 pounds of pressed yeast, which may be either baker's or brewer's yeast, add 30 cc of chloroform to it in a closed flask and allow it to stand at room temperature (20° C.) over night. By the morning the solid mass will have become fluid, and it should then be filtered through filter paper, allowing several hours for draining. To the filtrate add neutral lead acetate until no further precipitate forms and again filter. Precipitate the excess of lead from the filtrate with potassium oxalate and filter. To this filtrate add 25 cc of toluene and dialyze the mixture in a pig's bladder for two or three days, using running tap water. The dialyzed solution is colorless, perfectly clear after filtration, neutral to litmus, has a solid content of about one-half of 1 per cent, an ash content of a few hundredths of 1 per cent, will keep indefinitely in an ice box if a little toluene is kept on its surface to prevent the growth of micro-organisms, and is exceedingly active in inverting cane sugar. The invertase solution does not reduce Fehling's solution.

### THE KEEPING QUALITIES OF THE INVERTASE SOLUTION.

The stock solution of invertase prepared as described keeps perfectly. A quantity of it has been tested during a period of fifteen months and no change in its general appearance or inverting activity was noted. Three other preparations of less age have also shown no appreciable change. All of these solutions have been kept in an ice box, and toluene was added in excess of saturation in each case. Another preparation has been observed by H. S. Paine, of this Bureau, who found that the preparation did not change in its inverting activity during a period of three months. This sample was kept at room temperature (20° to 30° C.) during the day and in an ice box at night, thus duplicating the conditions which occur where many analyses are carried out each day with the use of the invertase solution.

### THE ROTATORY POWER OF THE INVERTASE.

The stock solutions of invertase have a slight rotatory power, and in a 400-mm tube they give a dextro-rotation of 1.0° V. In the method to be described further on the inversion of 95 cc of sugar solution is accomplished by 5 cc of the invertase solution, and therefore the correction to be applied for the rotation of the invertase solution is 0.05° when the reading is made in a 400-mm tube or 0.025° when a 200-mm tube is used. For all ordinary work these corrections are negligible, but they can always be exactly determined when accuracy requires their use.

### NUMERICAL FORMULA FOR THE DETERMINATION OF CANE SUGAR BY INVERTASE.

If $S$ denote the polarization of pure cane sugar before inversion and $I$ that after inversion, the numerical factor of inversion, which can be found by experiment, is $I/S$. This factor varies greatly with the temperature, due to the influence of the latter on the rotation of fructose. H. S. Paine and the author have measured this factor at 20° C., when invertase is used as the inverting agent on sugar solutions of 7 per cent strength and have found its value to be 0.317; similar experiments have given the factor 0.324 for the inversion by hydrochloric acid when the solution is kept at room temperature over night and read in the acid condition. For the same acid solution after neutralization the factor 0.317 was found, showing that it is the acidity which causes the difference between the factors for invertase and for the unneutralized acid solution. To check this point, the same quantity of hydrochloric acid was added to the solution which had been inverted by invertase and had given the factor 0.317; it was found that its rotation changed immediately to a greater negative value which corresponded to a factor 0.324, thus verifying the above conclusion in a different way. The inversions by invertase which gave the factor 0.317 were made in a solution which had been acidified with acetic acid, as invertase acts only in an acid solution; the two acid strengths of 0.01 and 1.0 normal were used and the factor was found to be the same for both, 0.317.

Other experiments have been made in which the solution was weakly acidified with other acids to produce an acid medium in which the invertase would act and for the inversion by invertase in these weak acid solutions (five ten-thousandths normal) the following values of the factor $I/S$ at 20° C. were found: Nitric, 0.315; sulphuric, 0.315; oxalic, 0.314; citric, 0.314; tartaric, 0.320; phosphoric, 0.315; arsenic, 0.315; hydrochloric, 0.315; and hydriodic, 0.315. The average of these values is 0.315, which is quite close to that just given for acetic acid and for the neutralized hydrochloric inversions, 0.317; a difference of 0.4° C. in the temperature at which the readings of the inverted solutions were made would account for this variation. Chas. A. Browne[a] has found the value 0.325 for the inversion by hydrochloric acid when the acid solution is read, and the value 0.317 after neutralization, numbers which are practically identical with those given here. Weber and McPherson[b] find at 20° C. the factor 0.317 for inversions by hydrochloric acid, correction being made for the influence of the acid.

L. M. Tolman[c] finds the factor 0.318 for acid inversions, the acid being corrected for, and states that, "if an inverting agent could be obtained which had no effect on the invert sugar, the question of concentration [influencing the factor] would practically be eliminated. But the elimination of this error would necessitate the determination of a new factor which would in all probability be 141.79 [i. e., 0.3179] or perhaps a little less." Invertase fulfils this requirement and its factor is 141.7.

It is thus certain that the factor for the inversion of cane sugar by invertase is considerably less than the factor for the usual hydrochloric acid inversion, that its value at 20° C. approximates 0.317 closely, and that the difference between the factors for invertase and hydrochloric acid is due to the effect of the acid on the rotatory power of invert sugar.

These experiments also prove that the hydrolysis of cane sugar by invertase is a complete one, for just as much invert sugar was formed by the invertase inversion as by that due to the strong hydrochloric acid. The usual formula for the per cent of cane sugar may, therefore, be used with the new factor 141.7 when the inversion is carried out by the use of invertase, namely:

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[a] U. S. Dept. Agr., Bureau of Chemistry, Bul. 110, p. 44.

[b] J. Amer. Chem. Soc., 1895, *17:* 324.

[c] J. Amer. Chem. Soc., 1902, *24:* 523.

$$\text{Per cent of cane sugar} = \frac{S-I}{141.7-\frac{T}{2}} \times 100.$$

## THE INFLUENCE OF ACIDS AND ALKALIS ON THE ACTIVITY OF INVERTASE.

Invertase acts only in an acid medium, and the slightest alkaline reaction stops its action instantly. Weak alkalinity does not destroy it, however, for if the acidity be restored the invertase resumes its original activity. But it is quite important that the medium be only weakly acid, for invertase is rendered inactive and even destroyed by acidities above hundredth-normal hydrochloric. As this acidity is itself rather weak, it seems best to avoid the use of strong mineral acids entirely in preparing the sugar solution for the action of invertase, and to use in their place a weak organic acid such as acetic, for invertase is quite active in acetic acid of 5 per cent strength.

In the accompanying figures there is given the measured activity of invertase in solutions of hydrochloric acid and of acetic acid. These activities were found by mixing, at 30° C., 100 cc of 0.2 normal (approximately 7 per cent) cane sugar solution with 5 cc of the appropriate strength of acid and 5 cc of a stock invertase solution, and measuring the velocity-coefficient ($k$) of the resulting inversion by the usual formula $k=\frac{1}{t}\log\frac{R_o-R_\infty}{R-R_\infty}$ where $R_o$ is the initial rotation of the solution, $R_\infty$ the value after complete inversion, $R$ the rotation at the time $t$ (expressed in hours), and log the common logarithm. Care was taken to make the samples alkaline before each reading of $R$ in order to stop the action of the invertase and complete the mutarotation of the invert sugar.

Figure 1 shows that invertase is active within only a very small range of acidity when the latter is due to a strong acid like hydrochloric, which is almost completely dissociated in solution, but if a weak acid like acetic is used, its dissociation is so slight that quite strong solutions can be used without bringing the actual acidity (i. e., hydrogen-ion concentration) up to a strength which is inimical to the action of invertase. As it would be difficult in practical work to adjust the acidity of the solutions very accurately, it is recommended that the solutions be all made acid to litmus with acetic acid, as even a considerable excess of acetic acid will do no harm.

## DIRECTIONS FOR MAKING THE ANALYSIS.

Dissolve 26 grams of the substance to be analyzed for cane sugar in water, clarify with the usual substances (neutral or basic lead acetate or alumina cream or kaolin) and make up to 100 cc volume at 20° C.

Filter and read the polarization of the filtrate, *S*, for a 200-mm tube. Remove the excess of lead from the filtrate, if lead has been used as clarifying agent, with sodium carbonate or potassium oxalate and filter. To 50 cc of the filtrate add acetic acid by drops until the reaction is acid to litmus, add 5 cc of the stock invertase solution and make up the volume to 100 cc. Add a few drops of toluene to the solution to prevent the growth of micro-organisms, shaking so as to saturate, and allow to stand at any temperature between 20° and 40° C. overnight. Under usual conditions about six hours' time is required to accomplish complete hydrolysis. In the morning bring the temperature to 20° and read the rotation of the solution, *I*, for a 400-mm tube. The percentage of cane sugar present is then calculated by the formula given on page 4. The percentage can also be found by the cuprous oxid reduction before and after inversion by the invertase.

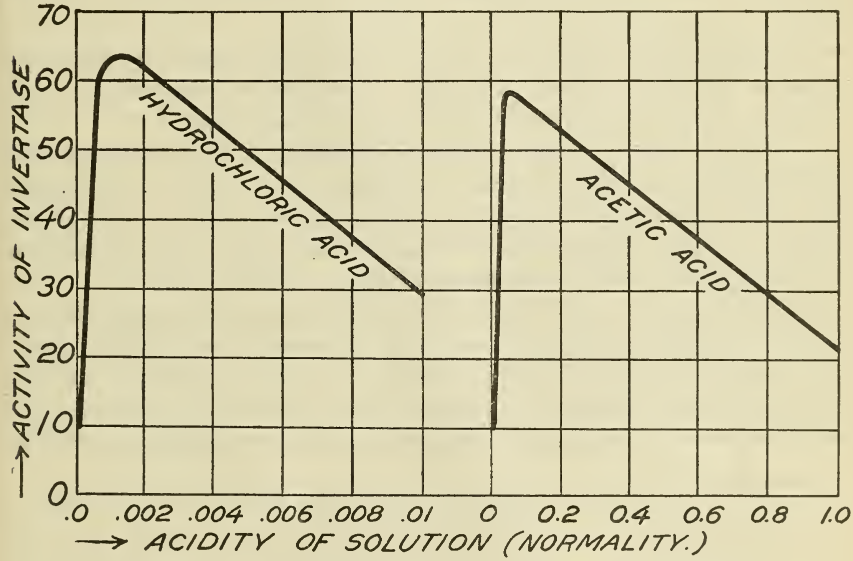

FIG. 1.—Influence of strong and weak acids on the activity of invertase.

## THE INFLUENCE OF MUTAROTATION ON THE INVERT POLARIZATIONS.

The temperature does not influence the rotation of glucose, but changes that of fructose greatly, and the change of rotation lags behind the change of temperature. This lag is due to the mutarotation of fructose and practically disappears in inversions by hydrochloric acid, for the reason that the acid is a strong katalyst of the mutarotation. In solutions inverted by invertase the lag for fructose which follows a change in the temperature of the solution lasts about ten minutes at 20° C., and care should therefore be used in reading the invertase inversions to see that the rotation remains constant for a few minutes. It is the writer's practice, after the invert reading is obtained, to add a pinch of cane sugar to the solution and observe its rotation for a few minutes; if it gradually changes, showing that the cane sugar added is being inverted, one is certain that the invertase is active and has accomplished a complete inversion overnight.

### TESTS OF THE INVERTASE METHOD.

1. *Test on pure cane sugar.*—Direct polarization ($S$), 100°; invert polarization, with hydrochloric acid, $-33.2°$; consequently the per cent sugar by Herzfeld's formula, $\frac{S-I}{142.7-\frac{T}{2}}\times 100$, is 100.4. The inversion by invertase gave $I=-31.9$, and the percentage by the above formula with the constant 141.7 for invertase, as described before, is 100.1. The polarization in all tests were made at 20° C.

2. *Test on Louisiana cane sirup.*—This sirup had been treated with sulphur dioxid in the process of manufacture. Direct polarization was 55.6°, invert polarization by hydrochloric acid, $-14.9°$, by invertase, $-13.8°$, giving the percentage of cane sugar by hydrochloric inversion as 53.1, and by invertase inversion 52.7 per cent.

3. *Test on bagasse.*—S. F. Sherwood, of this Bureau, recently obtained the following percentages of cane sugar in samples of Cuban bagasse, using the hydrochloric acid and also the invertase methods of inversion and determining the invert sugar for both methods by the change of polarization and also by the increased reduction of Fehling's solution:

*Determination of cane sugar in Cuban bagasse by the hydrochloric and the invertase methods. (Sherwood.)*

| Sample No. | Hydrochloric acid inversion. | | Invertase inversion. | |
|---|---|---|---|---|
| | Polarization. | Reduction. | Polarization. | Reduction. |
| | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* |
| 1 | 23.6 | 23.9 | 23.6 | 23.3 |
| 2 | 16.4 | 16.1 | 15.8 | 16.0 |
| 8 | 12.4 | 13.4 | 13.4 | 13.3 |
| 4 | 7.2 | 8.0 | 8.7 | 7.7 |

These three substances, pure cane sugar, cane sirup, and bagasse, show practically the same cane sugar percentage by the hydrochloric acid and the invertase methods. There will now be given an example of a very different type of analysis.

4. *Test on the sotol plant* (*Dasylirion texanum*).[a]—The soluble matter from 26 grams of this plant was dissolved to 100 cc and the polarization

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[a] This interesting plant occurs in the waste dry regions of New Mexico and Texas; a description of it is given in W. L. Bray's "The Vegetation of the Sotol Country in Texas." (Univ. of Texas, Bul. No. 60.)

$S$ found to be $-9.2°$. Inversion by hydrochloric acid gave $I= -16.5°$; by invertase $I= -9.7°$. The percentage of cane sugar determined by acid inversion was 5.5; by invertase inversion, 0.4. This analysis shows such a striking disagreement between the two methods that a further determination of the reducing sugar before and after the hydrolyses was made with Fehling's solution. The true carbohydrate composition of this plant was found by this method to be:

| | Per cent. |
|---|---|
| Reducing sugars | 2. 8 |
| Cane sugar (by invertase inversion) | 0. 7 |
| Unknown carbohydrate (by acid inversion) | 13. 3 |

Here, then, is a substance which contains only a trace of cane sugar, but a large quantity of a very easily hydrolyzed carbohydrate; further work on the nature of this unknown carbohydrate is in progress.

5. *Test on commercial glucose.*—A 26 per cent solution of "mixing glucose," which is prepared by the acid hydrolysis of starch, gave a rotation of 86.0° V. Inversion by hot hydrochloric acid changed this to 85.2°, by cold acid, 85.3°. Five drops of invertase solution were added to a third portion and after standing overnight its rotation was still 86.0°. The method of manufacture of this mixing glucose by hot acid hydrolysis makes it certain that no cane sugar can be present in it, and the method of analysis in which invertase is employed shows no cane sugar, but the usual acid methods give a slight decrease of rotation. Weber and McPherson[a] have particularly investigated this error in the acid methods of hydrolysis and devised a correction for it, but the invertase method is certainly preferable.

6. *Commercial glucose with added cane sugar.*—Commercial glucose is frequently flavored with a small quantity of cane sirup to make it more palatable, and it is then sold for table use. A determination of cane sugar in such "cane-flavored" glucose gave the following: Rotation of a 13 per cent solution of the sample in a 200-mm tube 75.0° V; rotation after inversion with hot hydrochloric acid, 72.7°; with cold hydrochloric acid, 73.8°; inversion by invertase, 74.0°. These results show that the acid methods indicate more cane sugar than does the invertase hydrolysis, and there is no question but that the invertase determination is more nearly correct.

## THE HYDROLYSIS OF RAFFINOSE BY INVERTASE.

The peculiar trisaccharid sugar raffinose, which occurs in cottonseed, in wheat, and in the sugar beet, is hydrolyzed by invertase, and the method for estimating cane sugar by inversion with invertase is accordingly not applicable in case raffinose is present. Raffinose is also hydrolyzed by hydrochloric acid, and therefore the acid hydrolysis has here no advantage over that by invertase. An experiment was performed in which a 10 per cent solution of very pure recrystallized raffinose, of specific rotation 123° (circular), was hydrolyzed with the stock invertase solution, and the rotation was found to change to 67.6° at 20° C., where it remained constant.

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[a] J. Amer. Chem. Soc., 1895, *17* : 312–320.

## THE ACTION OF INVERTASE ON OTHER ACID-HYDROLYZABLE SUBSTANCES.

The stock solution of invertase has been found to be entirely incapable of hydrolyzing lactose, maltose, starch, dextrin, cellulose, pentosans, amygdalin, or salicin. So far no action on any substances except cane sugar and raffinose has been detected, but it appears probable that the tetrasaccharid stachyose, which occurs in some plants, would be split into fructose and a trisaccharid.

## SUMMARY.

The enzym invertase accomplishes a complete inversion of cane sugar and is without action on starch, dextrin, maltose, lactose, pentosans, and natural glucosids. A procedure for preparing stock solutions of invertase from pressed yeast has been worked out and the solutions found to be permanent in inverting power, the measurements having been made now for a period of fifteen months. The invertase inverts only in an acid medium and the best acid to use is found to be acetic. Full directions for the method of analysis are given and the possible errors due to the slight rotatory power of the invertase solution and the mutarotation of the invert sugar are investigated and means taken to avoid them. The formula for the percentage of cane sugar is found to be $\frac{S-I}{141.7-\frac{T}{2}}\times 100$, where $S$ and $I$ are the direct and invert readings of the solution, $T$ the temperature, and 141.7 the inversion constant, which differs from that for the inversion by hydrochloric acid, i. e., 142.7. This difference is shown to be due to the influence of the acidity on the rotation of invert sugar, as the hydrochloric acid solutions give after neutralization the invertase constant 141.7. It is found that raffinose is hydrolyzed by invertase and that its specific rotation is thereby reduced from 123° to 67.6°; raffinose accordingly interferes with the estimation of cane sugar by the use of invertase, just as it also interferes when the inversion is carried out with hydrochloric acid. Determination of cane sugar in pure cane sugar, Louisiana molasses, and in bagasse by the invertase method of hydrolysis give percentages which agree with those obtained by the hydrochloric acid hydrolysis. The sotol plant (*Dasylirion texanum*) is shown to contain only a trace of cane sugar, but 13 per cent of an unknown carbohydrate which is being further investigated.